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BY

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# INFECTION OF FRUIT OF CITRUS BY PSEUDOMONAS CITRI [1]

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## INTRODUCTION

The present studies were made for the most part on citrus fruits grown in an isolated greenhouse, especially constructed for citrus-canker investigations, and situated near Washington, D. C. This paper gives findings regarding the invasion of the fruit tissues by the canker organism (*Pseudomonas citri* Hasse), the rate of multiplication of the bacteria in these tissues, and the resultant growth reactions of the fruit. In interpreting results and drawing deductions therefrom one must bear in mind that the environmental conditions in the greenhouse differ in many respects from those in commercial citrus plantings.

## METHOD OF INOCULATION

Most of the data from this investigation are of a quantitative nature, based on the number of visible lesions developing under a given set of conditions when a certain number of puncture wounds are inoculated. In routine puncturing of citrus leaves for such quantitative infection tests it has been found convenient to use a simple punch consisting of 10 or 20 pins or needles stuck through a cork. This method of quantitative testing with punctured grapefruit leaves was used in preference to poured agar plates because of the elimination of irregularities due to the growth on plates of contaminating organisms that have an inhibiting effect on *Pseudomonas citri*.

In the case of young fruit it was found that the puncturing instrument ruptured some of the oil glands. The exuding oil had a tendency to injure a portion of the adjacent tissue and to interfere with a normal infection reaction. A definite test of the matter was made by making a large number of punctures singly with a needle directly into the oil glands, and by making another group of punctures elsewhere on the same fruit and taking care to avoid the oil glands. Similar inoculum of *Pseudomonas citri* was applied to both groups, and the number of infections was noted after the lapse of sufficient time for their full development. (Fig. 1.) The results of such a test are given in Table 1.

It is evident that from the standpoint of careful experimental technic it is necessary to avoid making punctures into the oil glands of the friut. Puncturing of oil glands was avoided in the various experiments described in this paper by making punctures very carefully, one by one, under a magnifier.

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TABLE 1.—*Percentages of citrus cankers resulting on fruit from the inoculation of punctures into and between oil glands*

| Fruit and variety | Diameter of fruit | Cankers developed at punctures— | |
|---|---|---|---|
| | | Into oil glands | Between oil glands |
| | *Mm.* | *Per cent* | *Per cent* |
| Ponderosa lemon | 35 | 0 | 80 |
| Otaheite orange | 25 | 6 | 32 |
| Pineapple orange | 40 | 0 | 8 |

The inoculum was prepared from fresh, vigorously growing *Pseudomonas citri* cultures usually on potato plugs. These were checked closely to guard against diminution of virulence. Each batch was made up so as to be of approximately the same concentration. The inoculum was regularly applied to the fruit on moist cotton or cloth

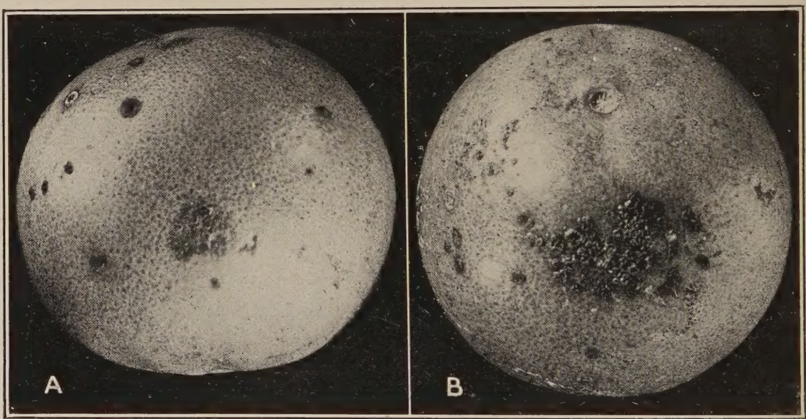


FIGURE 1.—Green grapefruit inoculated with a suspension of *Pseudomonas citri*. On one side (A) a group of punctures was made into the oil glands. On the other side (B) punctures were made between the oil glands. Extensive infection occurred in the latter instance, but none in the former. There are scattered stomatal infections where no punctures were made

swabs. These were left on under wrappings of several folds of paraffin paper, which were removed after a few days.

## INFLUENCE OF AGE OF WOUND ON AMOUNT OF INFECTION

To determine what influence the age of the wound at the time of infection has on the percentage of canker lesions that will develop, several groups of 50 punctures between oil glands were made on susceptible young fruits at four different times, over a period of 50 hours. Until the end of the 50-hour period the fruits were left dry and unwrapped. At that time inoculum of the same concentration was applied simultaneously to all groups of punctures on all fruits. The results are given in Table 2.

Evidently wounds as much as 8 hours old when kept dry are not very likely to become infected. Only 3 out of 13 fruits developed any infection through wounds of this age, and such infection was only

one-tenth to one-fifth as great as developed through fresh wounds on the same fruits. These three and one other fruit are the only ones in this test that developed stomatal [2] infections at apparently uninjured places. The fruits with stomatal infections were the youngest ones in the test, as evidenced by their comparatively small sizes for their respective varieties. All of this might indicate a state of greater tissue susceptibility for these fruits; but other fruits showing equal or higher percentages of infection for freshly made wounds failed to develop any infection in the wounds that were 8 hours old.

TABLE 2.—*Percentages of green-fruit punctures which developed cankers after being kept dry for different periods before inoculation*

| Fruit and variety | Diameter of fruit | Infection percentages, when the wounds dried for— | | | | Number of stomatal infections per fruit |
|---|---|---|---|---|---|---|
| | | Less than 1 hour | 8 hours | 26 hours | 50 hours | |
| | *Mm.* | | | | | |
| Pineapple orange | 46 | 20 | 0 | 0 | 0 | 0 |
| Do | 55 | 4 | 0 | 0 | 0 | 0 |
| Ruby orange | 38 | 8 | 0 | 0 | 0 | 0 |
| Otaheite orange | 20 | 20 | 2 | 0 | 0 | 3 |
| Do | 25 | 80 | 0 | 0 | 0 | 3 |
| Do | 36 | 12 | 0 | 0 | 0 | 0 |
| Duncan grapefruit | 42 | 20 | 4 | 0 | 0 | 1 |
| Ponderosa lemon | 21 | 44 | 6 | 0 | 0 | 5 |
| Do | 55 | 33 | 0 | 0 | 0 | 0 |
| Key lime | 30 | 48 | 0 | 0 | 0 | 0 |
| Rangpur lime | 40 | 50 | 0 | 0 | 0 | 0 |
| Citron | 34 | 2 | 0 | 0 | 0 | 0 |
| Do | 39 | 70 | 0 | 0 | 0 | 0 |

In another test the fruit was kept moist from the time of puncturing until inoculated. In this case infection occurred readily in wounds 24 hours old, but the percentages were lower than when the wounds were freshly made.

Perhaps here, as in the case of oil-gland punctures, the injury or killing of one or more layers of cells renders the substratum unfavorable for the development of infection. A fresh wound gives ready access to cells still living, but after the lapse of a few hours the progressive dying of exposed cells may present an obstacle to infection.

Under natural conditions the citrus-canker bacteria are disseminated mainly in water, and some of the greatest outbreaks of infection have occurred after violent storms that injured foliage and fruits. It might be inferred from these experimental tests that injuries such as thorn scratches, limb rubs, sand scurfs, or insect punctures or bites, occurring during dry weather, with the lapse of some hours before precipitation, would lead to comparatively little infection.

## INFLUENCE OF STRENGTH OF INOCULUM ON AMOUNT OF INFECTION

Tests were made of the effect of using very strong as compared with weaker grades of inoculum on wounded citrus fruits selected so as to be as nearly as practicable of the same size. The strongest inoculum, indicated as I/1, was made by using one 3-day-old potato-cylinder

[2] For convenience the term "stomatal infections" has been used to indicate the infections arising at points not wounded, although it is recognized that some of these may have been in reality at unrecognized wounds

culture of *Pseudomonas citri* in 200 c. c. of water. Dilutions of 1 in 20 and 1 in 400 were made from this. In Table 3 are given the results of one test carried out in this way. Others gave similar results.

TABLE 3.—*Percentages of wound infection of citrus fruits resulting from the use of various dilutions of inoculum*

| Fruit and variety | Diameter of fruit | I/1 inoculum | | I/20 inoculum | | I/400 inoculum | |
|---|---|---|---|---|---|---|---|
| | | Fruits | Average infection | Fruits | Average infection | Fruits | Average infection |
| | *Mm.* | *Number* | *Per cent* | *Number* | *Per cent* | *Number* | *Per cent* |
| Otaheite orange | 21–27 | 4 | 38 | 2 | 0.5 | 2 | 0.5 |
| Parson Brown orange | 34–37 | 1 | 25 | | | 1 | 2 |
| Double-flowered orange | 43 | 1 | 20 | | | 1 | 5 |
| Chinese lemon | 46 | 1 | 10 | 1 | 2 | | |

The strongest inoculum produced the highest percentages of infection, and the resulting cankers appeared first and reached full size soonest. The same inoculum applied at the same time to punctured grapefruit leaves gave 100 per cent infection from the I/20 dilution and 38 per cent from the I/400 dilution. In other tests punctured fruits of grapefruit have only rarely exceeded 50 per cent infection even with inoculum as strong as the I/1 used in this test. This indicates that very much stronger inoculum, approaching 400 times as strong, is required to produce a given amount of infection on wounded fruits than on wounded leaves of grapefruit. Similar findings have been made for commercial oranges, lemons, and limes. In natural outbreaks of canker in orchards, leaves seem to be much more readily infected than fruits.

These points are further shown in a test with Otaheite orange leaves and fruits using strong and weak inoculum, prepared as indicated above, of I/1 and I/400 strengths. (Table 4.) In recording results four stages of development of a canker spot are recognized: (1) Watery stage, the first distinctly visible water-soaked appearance; (2) pimple stage, small definite spots very slightly, if at all, raised; (3) blister stage, spots distinctly raised and enlarged but not broken open; and (4) erumpent stage, corky development approaching fully formed cankers. Table 4 indicates by stages the rate of development of the canker lesions as well as the percentages of infection on Otaheite oranges. The development throughout was somewhat slow because of rather low night temperatures in the greenhouse in the winter, when this test was made.

With each strength of inoculum the canker development was greater and faster on mature leaves than on either size of fruit, and on the smaller and younger fruits than on the somewhat larger and older ones. The strong inoculum brought about a quicker development of canker than did the weak inoculum on leaves and on both sizes of fruits. Comparing effects of I/1 inoculum on fruits with those of I/400 inoculum on the mature leaf, it is seen that the increase of four hundredfold in strength of inoculum was not sufficient to cause infection on these green fruits comparable with that produced on the leaf by the weaker strength of inoculum.

TABLE 4.—*Percentages of infection and rate of development of cankers on punctured fruits and leaves of Otaheite orange, following the use of strong and weak inoculum*

| Days after inoculation | Strong inoculum, I/1 | | | | | |
|---|---|---|---|---|---|---|
| | Mature leaf | | Small fruit (31 mm.) | | Large fruit (44 mm.) | |
| | Per cent infected | Stage | Per cent infected | Stage | Per cent infected | Stage |
| 2 | 0 | | 0 | | 0 | |
| 5 | 0 | | 0 | | 0 | |
| 7 | 20 | Pimple | 0 | | 0 | |
| 9 | 60 | Blister | 0 | | 0 | |
| 15 | ([a]) | | ([a]) | | ([a]) | |
| 19 | 100 | Erumpent | ([b]) | Watery | ([b]) | Watery. |
| 26 | 100 | do | 10 | Erumpent | ([b]) | Do. |
| 34 | 100 | do | 20 | do | 4 | Pimple. |
| 43 | 100 | do | 20 | do | ([c]) | |
| 50 | 100 | do | 40 | do | | |

| Days after inoculation | Weak inoculum, I/400 | | | | | |
|---|---|---|---|---|---|---|
| | Mature leaf | | Small fruit (34 mm.) | | Large fruit (47 mm.) | |
| | Per cent infected | Stage | Per cent infected | Stage | Per cent infected | Stage |
| 2 | 0 | | 0 | | 0 | |
| 5 | 0 | | 0 | | 0 | |
| 7 | 0 | | 0 | | 0 | |
| 9 | 15 | Blister | 0 | | 0 | |
| 15 | 75 | do | ([b]) | Watery | 0 | |
| 19 | 80 | do | ([b]) | do | 0 | |
| 26 | 85 | Erumpent | ([b]) | do | 0 | |
| 34 | 85 | do | ([c]) | | ([c]) | |
| 43 | 90 | do | | | | |
| 50 | 90 | do | | | | |

[a] No record made. [b] Few cankers. [c] Fruit dropped.

## RATE OF MULTIPLICATION OF PSEUDOMONAS CITRI IN FRUIT LESIONS

The method used for estimating the relative numerical increase of bacteria in inoculated fruit punctures was as follows: Punctures were made in numerous groups of 10 each on the fruit to be tested. Inoculation was made with cotton swabs in the usual way, the inoculum being strong enough to insure infection. Later at various time intervals a given number of the groups of punctures, regularly 6 comprising a total of 60 punctures, were removed with a flamed disk cutter in such way as to include the full thickness of the peel. These 6 uniformly cut disks were thoroughly teased out in 20 c. c. of sterile water. A portion of the resulting bacterial suspension, designated as d/1, was further diluted 1 in 20, and the dilutions were continued in the same ratio as far as might seem requisite. These several dilutions were then used to inoculate freshly made punctures on grapefruit leaves, regularly using 200 punctures on each of five leaves on each of two plants, making 2,000 punctures for a test of each dilution of each sampling. Records were made in due time of the number of cankers developing on the test leaves. The senior writer

has previously estimated[3] by this method that 2 to 4 infections developing in 2,000 punctures give evidence of something like 30 viable organisms per cubic centimeter of inoculum. This ratio of approximately 10 organisms per cubic centimeter of inoculum to 1 infection per 2,000 punctures holds very regularly up to about 300 infections per 2,000 punctures. Table 5 gives the results of one such test.

TABLE 5.—*Increase of Pseudomonas citri in a 34-mm. Otaheite orange fruit, as evidenced by the number of infections per 1,000 punctures developing on grapefruit leaves from inoculum prepared from samples taken at stated times from the orange fruit*

| Days after inoculation | Infections per 1,000 punctures developing from various dilutions of inoculum from samples from orange fruit | | | | |
|---|---|---|---|---|---|
| | d/1 | d/20 | d/400 | d/8,000 | d/160,000 |
| 0 | 0 | 0 | 0 | | |
| 2 | 80 | 6 | 0.5 | 0 | |
| 5 | 160 | 3 | .5 | 0 | 0 |
| 7 | 1,000 | 975 | 55 | 8 | 0.5 |
| 9 | 1,000 | 500 | 14 | 0.5 | 0 |
| 15 | 1,000 | 1,000 | 1,000 | 100 | 28 |
| 19 | 1,000 | 1,000 | 1,000 | 75 | 11 |
| 26 | 1,000 | 1,000 | 1,000 | 57 | 8 |

Evidently there was a very definite and considerable increase during the first 15 days in the number of bacteria in the sampled fruit punctures. From the fifteenth to the twenty-sixth day there was no evidence of continued increase, but there was a suggestion of possible decline in numbers. Tests made on the fifth and ninth days seem to be erratically low. Comparing the showing of the second day with that of the seventh, and the latter with that of the fifteenth, and noting the dilution of the original inoculum required to produce about the same effect on the test leaves, it is seen that there was an increase of perhaps two to four hundredfold of bacteria in the fruit wounds during each of these two periods. This means about eight generations of bacteria, if a regular geometrical rate of increase is maintained, during a 5-day period in the first case and during an 8-day period in the second. With the probability of considerable slowing down or actual reaching of a standstill two or three days before the fifteenth day, one may presume that most or all of the increase for the second period was also during a 5-day period.

The Otaheite orange fruit used in this test was the 34-mm. small fruit receiving the weak I/400 inoculum as shown in Table 4. The test was run at a somewhat low range of greenhouse temperature during the winter. This fruit did not give external evidence of infection until the fifteenth day, when there was hardly more than an indefinite watery appearance, and it did not develop more decided symptoms before dropping after the twenty-sixth day.

The sampling taken on the fifteenth day in the d/160,000 dilution gave infection of 28 out of 1,000 or 56 out of 2,000 wounds on grapefruit leaves. This would indicate about ten times 56 or 560 organisms

[3] FULTON, H. R. DECLINE OF PSEUDOMONAS CITRI IN THE SOIL. Jour. Agr. Research 19: 207–223. 1920.

per cubic centimeter for the inoculum used. The estimate for the d/1 inoculum would, therefore, be 160,000 times 560 or 89,600,000 organisms per cubic centimeter. If this is regarded as an even 90,000,000, the 20 cubic centimeters of this inoculum would have an estimated bacterial content of 1,800,000,000. These were from 60 inoculated fruit punctures, only a few of which showed the first suggestion of canker development. This would indicate an average of about 30,000,000 organisms in each incipiently infected wound at the end of the 15-day period. Tests with samples from similar spots made immediately after inoculation gave negative results even in the d/1 dilution by amethod that shouldhave shown the presence of as many as 30 organisms per cubic centimeter of inoculum, corresponding to 10 organisms from each sampled puncture. This indicates an increase ratio of something like 1 to 3,000,000.

Tests parallel to those shown in Table 5 for the small Otaheite orange fruit were made for the other five inoculated fruits and leaves shown in Table 4, except that samplings were not begun until the ninth day for the strong-inoculum series, and additional samplings were made on the thirty-fourth and fiftieth days for parts that had not dropped previously.

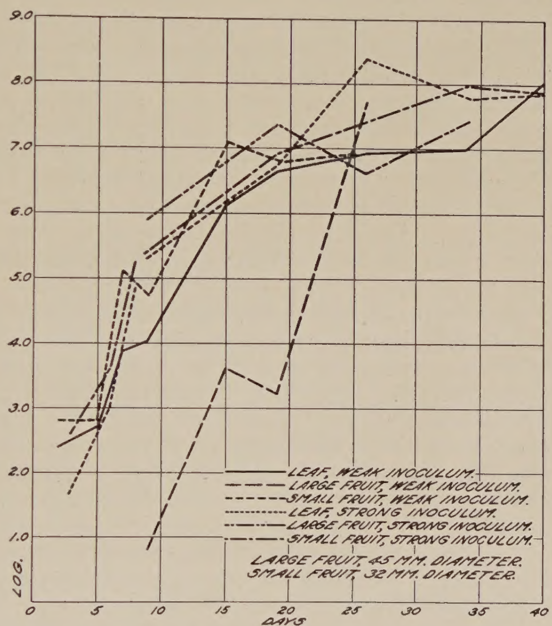


FIGURE 2.—Comparative rate of increase on a logarithmic basis of *Pseudomonas citri* in the tissue of large fruit, small fruit, and leaf of Otaheite orange following inoculation with strong (1/1) and weak (1/400) inocula. The breaks in the curves for the small fruit (strong inoculum) and the leaf (strong inoculum) indicate that the data were taken from two separate tests

In an earlier test made in September similar strong inoculum was used on a 34-mm. Otaheite orange fruit and on a mature leaf of the same plant, and three tests for bacterial increase were made on these at intervals during the first eight days. The data secured from this experiment seem to be concordant with the later results and are included in Figure 2.

The curves of Figure 2 were plotted to represent relative rates of increase of the bacteria in these six inoculated parts. The points on these curves were determined as follows: Relative estimates of the number of bacteria per infected puncture were made for each sampling by multiplying the dilution denominator by the number of infections produced per 2,000 leaf wounds; by multiplying this product by the factor 10 (average number of organisms per cubic centimeter of

inoculum to produce 1 infection per 2,000 leaf wounds), and by 20 (the number of cubic centimeters of inoculum); and by dividing the final product by 60 (the number of fruit punctures in the sample). An example of such a calculation is given on page 407.

To secure the greatest possible reliability for the estimates, two calculations were made for each sampling based on the results of two dilutions of inoculum selected as giving readings as nearly as possible within a range of 20 to 300 infection counts per 2,000 leaf punctures. The two estimates so obtained were averaged for the final estimate. The resulting number range from small to very large. To keep the graph within convenient size, the logarithms of the numbers were plotted rather than the numbers themselves. Since the multiplication of bacteria tends to be in geometrical progression, the plotting of logarithms gives a proper representation of such increase.

The indication from the increase curves of Figure 2 is that the bacteria multiplied with fairly equal rapidity in five of the six tests despite differences in host tissue and in strength of inoculum. The exception is the test of the larger fruit with the weak inoculum. Here there apparently was a slow start, followed by a very rapid increase to a maximum somewhat above that reached by four of the other five tests in the same 26-day period. The significant thing is that bacterial increase within the tissue should have been at so nearly the same rate while the development of visible lesions in the same material was so markedly different in both rate and extent of development, as has been shown in Table 4. For instance, on the ninth day for the strong inoculum neither of the fruits gave visible evidence of infection, while the leaf showed 60 per cent of the wounds at the blister stage. Inoculum prepared from samples of all three parts was of about equal potency on wounded grapefruit leaves. In the case of the three weak-inoculum tests there was an initial lag, which was overcome by the fifteenth day in two cases and by the twenty-sixth day in the third case. Generally speaking, approximately maximum development of bacteria was reached in about 15 days.

The test was repeated, using Otaheite orange of three sizes (40, 31, and 22 mm. in diameter) in comparison with mature leaves, and the same general results were obtained.

These experimental results raise a question as to what constitutes or measures susceptibility or immunity to citrus canker. Judging by external appearances as recorded in Table 4, one would say that the mature leaves of Otaheite orange are much more susceptible to wound infection than a 31-mm. fruit. But five samplings from the ninth to the fifty-sixth day gave indications of somewhat greater numbers of bacteria in the fruit than in the leaf for four of the five samplings. Even more striking is the comparison between the mature leaf and the small fruit receiving the weak inoculum. The development of an externally apparent canker is really a growth reaction of the host tissues. The presence of the causal organisms in sufficient numbers is a requisite for such development; but apparently under certain conditions the bacteria may be present in equal maximum number after having been equally active in multiplying within the tissue, and yet produce little or no canker reaction. In such cases the absence or weakness of canker development, usually taken to indicate resistance to the disease, does not seem to be due to any restraining

of bacterial activity, at least so far as their ability to grow and reproduce is concerned. This phenomenon constitutes what may be termed "quasi resistance" or "quasi immunity." This places emphasis on the importance of the reaction capacity of the host tissue rather than on the ability of the bacteria to flourish within the tissue as a determining factor in the production of canker lesions. The weak inoculum produced less visible effect than that four hundred times as strong (Table 4), but the results plotted in Figure 2 show that by the fifteenth day in two cases out of three the weak inoculations had reached approximately the same numerical level for bacterial invasion as the strong. While it might be assumed that a certain threshold number of bacteria must be present, or a certain quantity of bacterial metabolic activity must go on, in order to provide the proper stimulus for canker reaction in a given host-plant tissue, the present data indicate that the citrus tissue may be in a relatively nonresponsive condition without apparently offering any hindrance to *Pseudomonas citri* development, but in which the visible canker reaction is considerably limited or does not take place. Host tissues in different conditions of responsiveness may require different amounts of bacterial stimulus for a given effect, in which case the same amount of bacterial stimulus would be expected to produce different effects.

## RATE OF MULTIPLICATION OF PSEUDOMONAS CITRI IN GREEN FRUITS OF VARIOUS CITRUS VARIETIES

Using the method already described, tests were made on green citrus fruits of various varieties and various sizes. These tests were made during the summer months, and the higher range of temperature gave a more rapid rate of multiplication of canker bacteria than that shown in Figure 2. The samplings were made at more frequent intervals. Conditions were favorable for blue-mold rot, and the fruits often became infected through the sampling wounds in spite of all precautions. Whenever blue-mold rot set in, tests for viable canker bacteria gave negative results. Even before the sampled portion actually rotted there was a considerable reduction in the number of bacteria. The effect of blue-mold rot on the persistence of the canker organism is considered further in another section of the paper.

Logarithms to the first decimal place have been calculated to represent the average rate of increase of bacteria per inoculated puncture, and the results are given in tabular form in Table 6 for three separate experiments.

The first samplings made after inoculation gave somewhat irregular results. If such sampling is made immediately after the inoculum is applied, the showing for viable bacteria is abnormally high because of inclusion of the residue of inoculum. If made after about an hour, when the fruit has become surface dry, the showing is many times lower or even negative. Since the rate of drying and consequent dying of superfluous bacteria is very variable, the results obtained for starting figures are irregular and sometimes abnormally high. Usually only one fruit of a series was sampled at the time of inoculation, and the estimate obtained was assumed to hold for all in the series.

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TABLE 6.—*Relative logarithmic numbers of citrus-canker organisms estimated for infected punctures of green citrus fruits of various diameters and of leaves sampled at different times after inoculation, indicating the progressive numerical increase on a geometrical basis*

**Experiment A**

| Days after inoculation | Ponderosa lemon 37 mm. | Ponderosa lemon 62 mm. | Ponderosa lemon 87 mm. |
|---|---|---|---|
| 0 | 3.3 | 3.3 | 3.3 |
| 1 | 6.1 | 5.6 | 6.2 |
| 2 | | | 7.6 |
| 3 | 6.4 | 7.1 | 8.0 |
| 4 | | | |
| 5 | 5.4 | 5.9 | 6.4 |
| 8 | 2.4 | 7.2 | 4.9 |
| 10 | ([a]) | 6.4 | 5.9 |
| 14 | | 6.8 | 7.0 |
| 19 | | 6.5 | 1.9 |
| 24 | | 5 6 | [a] 3.6 |

**Experiment B**

| Days after inoculation | Parson Brown orange 42 mm. | Parson Brown orange 75 mm. | Pineapple orange 37 mm. | Pineapple orange 62 mm. | Satsuma orange (37 mm.) | Grapefruit leaf |
|---|---|---|---|---|---|---|
| 0 | 2.0 | 2.0 | 2.0 | 2.0 | 2.0 | 2.0 |
| 1 | 5.6 | 4.8 | 5.6 | 3.9 | 5.7 | 2.6 |
| 2 | 4.7 | 5.0 | 4.2 | 5.6 | 5.2 | 4.1 |
| 3 | 2.6 | 4.2 | 4.3 | 3.0 | 5.7 | |
| 4 | 2.8 | 1.8 | 5.2 | 2.6 | 4.9 | 4.2 |
| 6 | ([a]) | ([a]) | 5.5 | ([a]) | 5.5 | 4.9 |
| 8 | | | 4.8 | | 4.8 | 6.1 |
| 10 | | | 4.7 | | | 5.4 |
| 13 | | | | | 4.6 | 5.5 |

**Experiment C**

| Days after inoculation | Key lime (34 mm.) | Walters grapefruit (48 mm.) | Grapefruit leaf |
|---|---|---|---|
| 0 | 0.6 | 0.6 | 0.6 |
| 1 | | | |
| 2 | 6.2 | 6.0 | 4.6 |
| 3 | | | |
| 4 | ([a]) | ([a]) | 4.6 |
| 5 | | | |
| 7 | | | 5.9 |
| 10 | | | 6.4 |

[a] Fruit affected with blue-mold decay.

For the three Ponderosa lemons the rate of increase was much the same up to the fifth day, when the smallest one began to show a decline, the fruit being invaded by Penicillium. The 87-mm. fruit was similarly affected after the fourteenth day. This largest fruit did not develop any external evidence of canker reaction during the 24 days. The 62-mm. lemon developed 50 per cent of fully erumpent cankers during the test. The smallest (37 mm.) fruit showed 10 per cent of erumpent spots before its premature loss, and undoubtedly would have shown the greatest amount of canker reaction if it had persisted.

The two sizes of Parson Brown oranges and the two of Pineapple oranges showed definite increases to about the same general level. Unfortunately, three of these four fruits succumbed to blue-mold rot after the fourth day and before visible cankers could be expected. The remaining 37-mm. Pineapple orange developed 5 per cent erumpent cankers during the first 10 days. The 37-mm. Satsuma orange did not develop any external evidence of infection during the 19-day period. Field observations in canker-infested territory have shown the very high resistance amounting almost to immunity of Satsuma fruits to natural infection. In the present experiment the rate of increase in Satsuma fruit was fully as rapid as in the Pineapple orange of the same size, the latter developing normal symptoms of canker and the former none. The invasion through wounds and the subsequent multiplication of canker bacteria seem to have been closely parallel. One variety reacted in such a way as to form cankers, but the other variety did not visibly react. The grapefruit leaf in the same experiment gave lower tests in early stages than the orange fruits, but the maximum finally reached was approximately the same. The leaf by the thirteenth day had developed cankers of the blister stage at 100 per cent of the wounds.

The last section of Table 6 (experiment C) shows very rapid increase during two days in wounded fruits of key lime and Walters grapefruit, and the increase in wounded grapefruit leaf is given for comparison. Unfortunately, the two fruits were soon lost from blue-mold rot. The grapefruit leaves showed 90 per cent of visible canker in the blister stage on the tenth day. The possibility of wound infection without external reaction is thus demonstrated for a representative series of green citrus fruits. With increase in size of fruit beyond a certain point the development of external visible lesions is hindered, but the bacteria multiply practically as rapidly as when typical cankers are formed.

## RATE OF MULTIPLICATION OF PSEUDOMONAS CITRI IN MATURE FRUITS OF VARIOUS CITRUS VARIETIES

Some of the inoculated fruits used in previous tests were approaching full size, but the peel was still green. It seemed desirable to test fruit in a more matured condition. Tests were made on fully colored fruit still attached to the trees in the greenhouse. The results are given in Table 7. A comparison with Table 6 shows very little difference in rate of increase between green and mature fruit. As mature fruit soon became infected with blue-mold rot, the tests ended prematurely.

TABLE 7.—*Relative logarithmic numbers of citrus-canker organisms estimated for each infected puncture of ripe citrus fruits sampled at different times after inoculation, indicating the progressive numerical increase on a geometrical basis*

| Experiment A | | | | Experiment B | | |
|---|---|---|---|---|---|---|
| Days after inoculation | Pineapple orange | Duncan grapefruit | Ponderosa lemon | Days after inoculation | Pineapple orange | Ponderosa lemon |
| 0 | 2.8 | 2.8 | 2.8 | 0 | 1.3 | 0.9 |
| 1 | 7.9 | 6.0 | 7.7 | 1 | 4.2 | 3.9 |
| 2 | 6.8 | 7.4 | 6.6 | 2 | 5.2 | 6.2 |
| 3 | | | | 3 | 5.0 | 7.3 |
| 5 | 5.3 | 2.5 | 5.4 | 6 | 2.3 | 4.0 |

Mature fruits of orange, grapefruit, and lemon from the market were tested at two different times. In one instance the fruit was lost from decay after the fifth day; in the other it was held over a 28-day period and sampled every five days. In both tests the orange and the lemon gave negative results at every sampling after the first, which was made just after inoculation. The grapefruit in each case gave low estimates on the fifth day, indicating persistence with little if any multiplication. In the longer test the same condition was shown by the grapefruit on the tenth, fifteenth and twenty-first days, with negative results on the twenty-eighth day, the last testing. In still another experiment a mature grapefruit from the market was inoculated through wounds in the usual way, and 74 days later a test indicated something like 32,000 bacteria per puncture. This fruit had been held without intervening sampling and had not developed external lesions.

There is apparently a very marked difference in the behavior of the canker organism following inoculations in the peel of mature fruit after removal from the tree as compared with its behavior in the peel of mature fruit still on the tree. Possibly changes in the physiological condition of the fruit resulting from its removal from the tree are responsible for this difference. It is to be noted that in the cases of Diplodia and Phomopsis stem-end rots the advance of rot into the peel normally does not occur until some days or weeks after the fruit is removed from the trees, although there is abundant evidence that incipient infection already exists. Here the senescent changes in the peel favor the development of fungi having saprophytic tendencies; it is not inconsistent to presume that these changes would in equal degree hinder the development of an organism having definitely parasitic habits like *Pseudomonas citri*.

## PERSISTENCE OF BACTERIA IN OLD CANKERS ON MATURE FRUITS

The question arises about the viability of *Pseudomonas citri* in typical canker lesions of mature fruits that originated while the fruit was young and green. In the greenhouse experiments certain fruits were inoculated while young and developed typical canker lesions. Six to seven months later, when these fruits were fully mature, tests were made on punctured grapefruit leaves in the usual way for evidence of viable canker bacteria. The test included two fruits of Pineapple orange, three fruits of Otaheite orange, two fruits of Pon-

derosa lemon, two fruits of Rangpur lime, one fruit of Temple orange, and one fruit of Duncan grapefruit. In every case the results were negative. (Fig. 3.)

From time to time fruits with well-developed cankers have been intercepted by quarantine inspectors and submitted for diagnosis. The obtaining of viable *Pseudomonas citri* from such specimens has been uncertain, although in a few instances successful cultures of the canker organism have been made from such material. (Fig. 4.) Infected fruits were too limited in number for a definite determination of the time limits of viability of the organisms in the lesions. This doubtless varies greatly with conditions.

## EFFECT OF PENICILLIUM ROT ON VIABILITY OF PSEUDOMONAS CITRI

It was noted earlier in this paper that development of Penicillium rot in a fruit under test is regularly followed by a decrease in the number of viable canker bacteria recoverable. To test the matter definitely, mature orange fruits on the tree were puncture-inoculated at several locations with the canker organism. One day later *Penicillium digitatum* was introduced at one place on the fruit. Two days after this, when the softening from the blue-mold rot was apparent, tests were made from groups of punctures very near the advancing edge of the rot, but where the tissue had not yet softened, and also from punctured areas on the opposite side of the fruit. In one experiment the average of five tests from near the edge of the rotted area gave 5 infections per 1,000 grapefruit-leaf punctures for the d/1 inoculum, and a similar average for material from the opposite side of the fruit was 975 infections per 1,000 test punctures for a similar dilution of inoculum. In a second experiment samples taken from areas recently invaded by Penicillium gave negative results, while material taken at a distance gave an average of 403 infections per 1,000 test punctures.

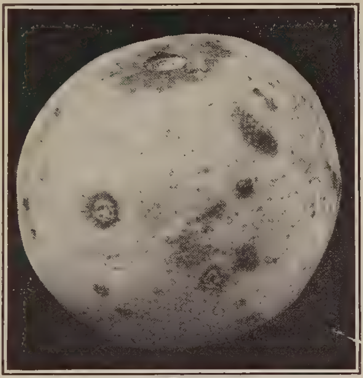

FIGURE 3.—Canker lesions on a mature Pineapple orange, the result of inoculation of the young fruit six months previously. These old cankers did not give evidence of containing viable *Pseudomonas citri* when culture and inoculation tests were made

## INFLUENCE OF SIZE OF FRUIT ON DEVELOPMENT OF CANKER

In previous tests estimates were made quantitatively for the presence of the canker bacteria in the host tissues, and estimates of their numbers were made regardless of whether there were externally apparent lesions. Such testing requires much time and material. In ordinary practice reliance is put on the development of externally visible lesions to determine the fact and extent of infection. In the experiments that follow, this observational method was used to determine the range of fruit size or age in which infection is possible, either through wounds or without wounding, and the size of fruits giving the best development of lesions.

The method used may be illustrated by the following experiment: Four Homosassa oranges of approximately the same size, averaging 30 mm. in diameter, were punctured carefully between the oil glands, 50 punctures in each group, inoculated with standard inoculum on a swab, and wrapped. About 7 to 10 days later a new group of punctures was made in a different location on the same fruits, inoculum was applied, and the fruits were remeasured. The same procedure was followed until five successive tests had been made on these fruits, the last being when their average size was 50 mm. Close records were kept of the starting of visible infection at any group of wounds, or on the adjacent unwounded but inoculated surface.

In this test the average infection at the outset, at 30 mm. average size, was 47 per cent of erumpent lesions. The greatest average percentage of infections for any inoculation was 82 per cent at the 36 mm. average size, and these lesions reached a greater size than the others. At an average size of 48 mm. the lesions were still fairly numerous, averaging 41 per cent, but they were quite small, and none became erumpent. At 50 mm. average size there was only 9.5 per cent of infection, and the lesions were barely discernible as such. It thus appears, as judged by ordinary standards, that Homosassa oranges, under the conditions of this test, manifested greater susceptibility to canker infection through wounds at a size of about 36 mm. (nearly 1½ inches) in diameter than at smaller or larger sizes. The upper size limits of susceptibility to wound infection seemingly had been almost reached at the end of the test at an average size of 50 mm. (2 inches). In this test, fruits developing stomatal infections were more closely limited in size range, the smallest fruits showing any infection being the 29 mm. size and the largest 35 mm., with an average size of 32 mm. and an average of eight infections per fruit, all well developed. The beginning size for stomatal infections on this variety is not indicated by this test, and the optimum size is very likely smaller than the initial sizes used. Since inoculations were repeated three or four times after the fruits had passed the 35 mm. size, it may be safely concluded that this is about the upper limit of susceptibility for stomatal infection of this variety under the conditions of this experiment.

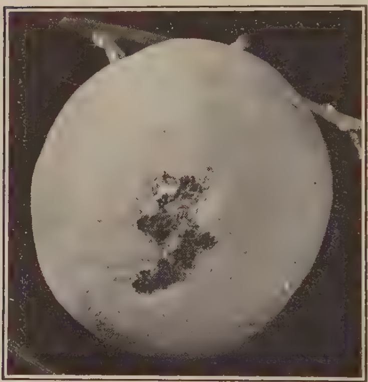

FIGURE 4. -Orange infected with canker, intercepted at San Francisco in the baggage of a passenger from Japan. Cultures or inoculation material from old lesions on such mature fruits may or may not give evidence of viability of *Pseudomonas citri*

In the same general way tests were made on a considerable number of varieties of green citrus fruits in the greenhouse. (Fig. 5.) Since the supply of such fruits was limited, it was not usually possible to select a group of the same size for inoculation at the same time. Inoculations were therefore made on fruits of any suitable size, proper measurements and records being made. It is recognized that size is by no means an exact indication of age, since individual fruits

may grow at different rates even when of the same variety, and the different varieties and classes of citrus fruits vary much in rate of growth and maximum size attained. This testing of varieties extended over three seasons, and naturally the various fruits used were subjected to a variety of growth conditions. An attempt was made to make inoculations as uniformly as possible, and with inoculum strong enough to produce a maximum of infection. Parallel inoculations were always made on punctured grapefruit leaves and always yielded practically 100 per cent of infection. Difficulty was experienced in getting records on fruits smaller than about 18 mm., because the injury caused by the puncturing and the covering of moist wrappings seemed to induce dropping. Records of infection were made after sufficient time had elapsed for infection to reach a


FIGURE 5.—A.—Ponderosa lemon. Five rows of 10 punctures each (partly showing) were made and inoculated when the fruit was 50 mm. in diameter. These developed 82 per cent infection. The circular group of 50 punctures at the left was made and inoculated when the fruit had reached a diameter of 65 mm., but no infection resulted. B.—Navelencia orange. The circular group of 50 punctures at the right side of the fruit was made and inoculated when the fruit was 28 mm. in diameter, and developed 30 per cent infection at wounds. There were also a few stomata lesions. Another group of 50 punctures (near the lower side of the fruit) was made and inoculated when the fruit had reached a diameter of 43 mm. These did not become infected

maximum, and distinction was made between wound and stomatal infections.

Table 8 gives the summarized results of these tests with particular reference to the relationship of fruit size to development of infection. In interpreting these results it must be borne in mind that the lower limits of infectable range could not be definitely determined because of the difficulty in getting very small sizes to persist after being inoculated. The poor condition of some that remained may have interfered with normal development of infection. If the fruits were not punctured, more of them remained attached, and so the test range could be carried lower for stomatal than for wound infections. It is noteworthy that the sizes that gave the greatest amounts of visible infection were usually at some distance from either the upper or the lower limits of the range tested, when any considerable number of tests were made for any variety. Toward the upper limits of infection the reaction lessened progressively from visible excres-

cences to mere external yellowing and finally to slight internal discoloration. It must be emphasized that the results in Tables 8 and 9 are not finally conclusive, and caution must be exercised in making any general deductions from them. In some instances the numbers of fruits tested were too low for reliable results. Numerical estimates based on the inoculated leaf puncture method of testing have shown the active invasion of fruits of Valencia orange to be about equal to Pineapple orange in one specific experiment, and that of Washington Navel to be distinctly less. In the same way invasion of fruit of Royal grapefruit was proved to take place, but in a less degree than in Marsh grapefruit. A single test to determine invasion of fruit of Nagami (oblong) kumquat gave negative results under rather unfavorable conditions for the test, due to the usually quick rotting of wounded kumquats.

TABLE 8.—*Results of inoculating fruits of various citrus varieties with Pseudomonas citri, showing the sizes most readily infected*

| Fruit and variety | Data concerning wound infections | | | | | Datâ concerning stomatal infections | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Tests | Size range tested | Infected fruits | Size range infected | Optimum size for infection | Tests | Size range tested | Infected fruits | Size range infected | Optimum size for infection |
| | *Number* | *Mm.* | *Number* | *Mm.* | *Mm.* | *Number* | *Mm.* | *Number* | *Mm.* | *Mm.* |
| Pineapple orange | 54 | 15–58 | 36 | 19–58 | 40 | 60 | 8–58 | 10 | 22–43 | 31 |
| Homosassa orange | 30 | 17–54 | 23 | 17–54 | 36 | 36 | 17–54 | 3 | 29–35 | 30 |
| Parson Brown orange | 34 | 15–61 | 20 | 21–57 | 33 | 34 | 15–61 | 3 | 22–26 | 22 |
| Mediterranean Sweet orange | 11 | 21–49 | 4 | 21–48 | 25 | 15 | 16–49 | 1 | 21 | 21 |
| Valencia (Lue) orange | 9 | 25–49 | 0 | -------- | -------- | 9 | 25–49 | 0 | -------- | -------- |
| Navelencia orange | 8 | 25–54 | 3 | 25–32 | 27 | 8 | 25–54 | 2 | 28–32 | 28 |
| Ruby orange | 4 | 38–58 | 1 | 38 | 38 | 4 | 38–58 | 0 | -------- | -------- |
| Washington Navel orange | 3 | 18–49 | 0 | -------- | -------- | 3 | 18–49 | 0 | -------- | -------- |
| Satsuma orange | 37 | 18–51 | 12 | 18–40 | 28 | 50 | 11–51 | 3 | 28–35 | 33 |
| King orange | 19 | 20–38 | 6 | 22–38 | 30 | 20 | 20–38 | 0 | -------- | -------- |
| Tangerine orange | 17 | 16–42 | 2 | 23–28 | 28 | 22 | 11–42 | 1 | 24 | 24 |
| Temple orange | 4 | 38–60 | 0 | -------- | -------- | -------- | -------- | 0 | -------- | -------- |
| Otaheite orange | 39 | 9–70 | 22 | 14–47 | 24 | 46 | 11–70 | 3 | 20–25 | 25 |
| Double-flowered orange | 14 | 25–48 | 6 | 33–46 | 42 | 17 | 19–48 | 0 | -------- | -------- |
| Myrtleleaf orange | 13 | 16–45 | 3 | 25–36 | 30 | 13 | 16–45 | 0 | -------- | -------- |
| Willowleaf orange | 3 | 30–40 | 1 | 30 | 30 | 3 | 30–40 | 0 | -------- | -------- |
| Duncan grapefruit | 28 | 25–66 | 11 | 25–53 | 40 | 33 | 8–66 | 8 | 25–42 | 38 |
| Walters grapefruit | 11 | 28–65 | 3 | 28–48 | 48 | 13 | 15–65 | 2 | 40–48 | 40 |
| Royal grapefruit | 3 | 28–46 | 0 | -------- | -------- | 3 | 28–46 | 0 | -------- | -------- |
| Eureka lemon | 9 | 30–45 | 3 | 33–36 | 33 | 11 | 21–45 | 0 | -------- | -------- |
| Lisbon lemon | 10 | 21–40 | 1 | 38 | 38 | 9 | 21–40 | 0 | -------- | -------- |
| Kenedy lemon | 10 | 23–38 | 2 | 27–35 | 31 | 11 | 17–38 | 0 | -------- | -------- |
| Villafranca lemon | 6 | 27–45 | 1 | 27 | 27 | 7 | 27–45 | 0 | -------- | -------- |
| Lamb lemon | 5 | 28–48 | 0 | -------- | -------- | 3 | 18–49 | 0 | -------- | -------- |
| Chinese lemon | 19 | 30–53 | 8 | 30–46 | 36 | 19 | 38–53 | 1 | 35 | 35 |
| Rough lemon | 8 | 21–33 | 0 | -------- | -------- | 14 | 18–33 | 0 | -------- | -------- |
| Ponderosa lemon | 45 | 17–92 | 25 | 21–88 | 35–75 | 54 | 11–92 | 9 | 21–70 | 56 |
| Key lime | 20 | 14–31 | 13 | 17–30 | 26 | 32 | 9–31 | 3 | 20–28 | 24 |
| Rangpur lime | 36 | 18–55 | 22 | 18–48 | 27 | 51 | 8–55 | 3 | 22–24 | 23 |
| Sylhet lime | 16 | 15–35 | 6 | 19–33 | 22 | 16 | 15–35 | 1 | 19 | 19 |
| Suntara lime | 8 | 18–32 | 6 | 18–29 | 23 | 8 | 18–32 | 1 | 21 | 21 |
| Bearss lime | 11 | 17–37 | 7 | 17–36 | 20 | 11 | 17–37 | 0 | -------- | -------- |
| Kusaie lime | 18 | 11–32 | 3 | 18–32 | 20 | 22 | 7–32 | 0 | -------- | -------- |
| Dominican lime | 18 | 14–36 | 3 | 16–26 | 21 | 21 | 9–36 | 0 | -------- | -------- |
| Woglum lime | 2 | 34–36 | 0 | -------- | -------- | 2 | 34–36 | 0 | -------- | -------- |
| Tahiti lime | 7 | 15–35 | 0 | -------- | -------- | 10 | 12–35 | 0 | -------- | -------- |
| Nagami kumquat | 17 | 12–23 | 0 | -------- | -------- | 17 | 12–23 | 0 | -------- | -------- |
| Citron | 9 | 34–44 | 3 | 34–39 | 39 | 9 | 34–44 | 0 | -------- | -------- |
| Citrus excelsa | 5 | 12–28 | 1 | 13 | 18 | 6 | 12–28 | 0 | -------- | -------- |

In Table 9 data from the same tests are used as a basis for quantitative expressions of infection for the various varieties. In the tabulation are included only those fruits that fall within the known infectible range of size for the particular variety. Not all fruits within such range actually developed infection in the tests. Several varieties in which neither wound nor stomatal infection was secured are omitted from this table. However, it is to be supposed that some of these might react under more suitable conditions or with more extended testing. Since the bases on which percentages of infection are calculated are reduced by this attempted elimination of sizes that may be immune, the resulting showing of infection is higher and supposedly a more correct index to the relative susceptibility of the various species. But caution must be exercised in drawing general conclusions from data having the unavoidable limitations of these. It must be recalled that what is here termed "susceptibility" is evidenced and measured by the outwardly visible reaction of the host tissues to the bacterial invasion. It has been shown in other cases that the bacterial invasion takes place just as truly, but the host tissues do not respond sufficiently to show any external effect. The present data do not take into account such invisible invasion.

TABLE 9.—*Results of inoculating infectible fruits of various citrus varieties with Pseudomonas citri*

| Fruit and variety | Data concerning wound infections | | | | | | Data concerning stomatal infections | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | Fruits of susceptible sizes | | | Infected fruits | | | Fruits of susceptible sizes | | | Infected fruits | |
| | Fruits | Infected | | Punctures | Lesions | Punctures with lesions | Fruits | Infected | | Lesions | Average lesions per fruit |
| | *Number* | *Number* | *Per cent* | *Number* | *Number* | *Per cent* | *Number* | *Number* | *Per cent* | *Number* | *Number* |
| Pineapple orange | 52 | 36 | 69 | 1,800 | 614 | 34 | 43 | 10 | 23 | 85 | 9 |
| Homosassa orange | 30 | 23 | 77 | 1,150 | 415 | 36 | 11 | 3 | 27 | 30 | 10 |
| Parson Brown orange | 31 | 20 | 59 | 1,000 | 176 | 18 | 12 | 3 | 25 | 31 | 10 |
| Mediterranean Sweet orange | 11 | 4 | 36 | 200 | 32 | 16 | 5 | 1 | 20 | 1 | 1 |
| Navelencia orange | 3 | 3 | 100 | 150 | 110 | 73 | 3 | 2 | 67 | 30 | 15 |
| Ruby orange | 1 | 1 | 100 | 50 | 4 | 8 | ------ | 0 | ------ | ------ | ------ |
| Satsuma orange | 35 | 12 | 34 | 600 | 99 | 17 | 49 | 3 | 6 | 27 | 9 |
| King orange | 19 | 6 | 32 | 300 | 27 | 9 | ------ | 0 | ------ | ------ | ------ |
| Tangerine orange | 10 | 2 | 20 | 100 | 6 | 6 | 6 | 1 | 17 | 10 | 10 |
| Otaheite orange | 29 | 22 | 76 | 1,100 | 221 | 20 | 12 | 3 | 25 | 10 | 3 |
| Double-flowered orange | 12 | 6 | 50 | 300 | 56 | 19 | ------ | 0 | ------ | ------ | ------ |
| Myrtleleaf orange | 10 | 3 | 30 | 150 | 9 | 6 | ------ | 0 | ------ | ------ | ------ |
| Willowleaf orange | 1 | 1 | 100 | 50 | 10 | 20 | ------ | 0 | ------ | ------ | ------ |
| Duncan grapefruit | 23 | 11 | 48 | 550 | 157 | 29 | 13 | 8 | 62 | 53 | 7 |
| Walters grapefruit | 7 | 3 | 43 | 150 | 45 | 30 | 6 | 2 | 33 | 25 | 13 |
| Lisbon lemon | 4 | 1 | 25 | 50 | 26 | 52 | ------ | 0 | ------ | ------ | ------ |
| Eureka lemon | 5 | 3 | 60 | 150 | 26 | 17 | ------ | 0 | ------ | ------ | ------ |
| Kenedy lemon | 8 | 2 | 25 | 100 | 64 | 64 | ------ | 0 | ------ | ------ | ------ |
| Villafranca lemon | 2 | 1 | 50 | 50 | 13 | 26 | ------ | 0 | ------ | ------ | ------ |
| Chinese lemon | 18 | 8 | 44 | 400 | 56 | 14 | 9 | 1 | 11 | 2 | 2 |
| Ponderosa lemon | 43 | 25 | 58 | 1,250 | 541 | 43 | 39 | 9 | 23 | 71 | 8 |
| Key lime | 20 | 13 | 65 | 650 | 116 | 18 | 20 | 3 | 15 | 13 | 4 |
| Rangpur lime | 32 | 22 | 69 | 1,100 | 425 | 39 | 11 | 3 | 27 | 9 | 3 |
| Sylhet lime | 16 | 6 | 38 | 300 | 81 | 27 | 3 | 1 | 33 | 5 | 5 |
| Suntara lime | 7 | 6 | 86 | 300 | 66 | 22 | 4 | 1 | 25 | 1 | 1 |
| Bearss lime | 11 | 7 | 64 | 350 | 52 | 15 | ------ | 0 | ------ | ------ | ------ |
| Kusaie lime | 17 | 3 | 18 | 150 | 105 | 70 | ------ | 0 | ------ | ------ | ------ |
| Dominican lime | 17 | 3 | 18 | 150 | 14 | 9 | ------ | 0 | ------ | ------ | ------ |
| Citron | 9 | 3 | 33 | 150 | 43 | 29 | ------ | 0 | ------ | ------ | ------ |
| Citrus excelsa | 1 | 1 | 100 | 50 | 3 | 6 | ------ | 0 | ------ | ------ | ------ |

## INOCULATION OF FRUIT ON ORCHARD TREES

On account of the vigorous eradication campaign against citrus canker, it has not been possible for the writers to make test inoculations on orchard trees except in one instance. These few trees were outside of a commercial citrus region, in territory in which the eradication work was at the time suspended. The orange trees were seedlings, about 12 years old when the inoculations were made in May. There was already considerable old and fresh canker infection on the leaves of the lower limbs, but none was observed on the fruits. Fruits for inoculation were selected on the topmost branches above the drip from infected leaves. The fruits were punctured between the oil glands, 100 punctures each. Forty fruits were used, ranging in size from 18 to 38 mm., all but four being 25 mm. or larger. Strong inoculum was prepared by teasing well-developed leaf lesions in water until it looked turbid. The inoculum was applied on cotton swabs, and the fruit was wrapped with waxed paper. Punctured leaves were also inoculated on the same tree.

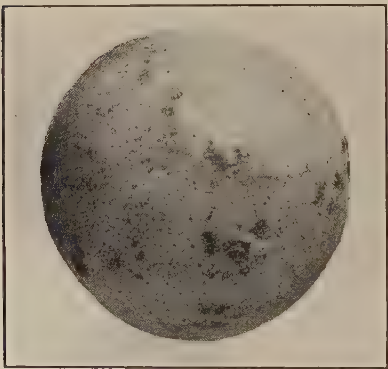

FIGURE 6.—Moist-chamber inoculation on detached green orange, 45 mm. in diameter

After one month, 16 fruits showed infection, all at wounds; 4 of them were in the 25-mm. class and averaged 4 infections per fruit, and 5 were in the 32-mm. class and averaged 3 infections per fruit. The greatest number of infections per fruit was 14 on a fruit of the 32-mm. class; the next was 12 on a fruit of the 38-mm. class; and the next was 6 on a fruit of the 25-mm. class. Of the 16 infected fruits, 7 had 1 infection per fruit. There was no increase in infection during the following three months. No stomatal infection developed on the inoculated fruits or on other fruits on the tree that had a good chance to receive washings from infected leaves. The artificially inoculated leaves developed 100 per cent infection.

While this test was too small in scope for any definite conclusions to be drawn, the behavior of the fruits growing naturally on large trees in this one instance was in general agreement with the experiments in the greenhouse in which green fruits on the trees were inoculated in a similar way.

## INOCULATION OF DETACHED GREEN FRUITS IN MOIST CHAMBERS

Since the numbers and varieties of green citrus fruits available for inoculation in the greenhouse were quite limited, attempts were made to broaden the range by using young fruits that had been removed from the trees in Florida and sent to Washington. (Fig. 6.) These were punctured in the usual way, inoculated by dipping in a suspension of *Pseudomonas citri*, and placed in covered dishes lined with moist filter paper, care being taken to avoid too much

moisture. Small fruits, in general less than 20 mm. in diameter, were apt to decay quickly. The larger ones developed canker lesions rather more readily than did those on trees in the greenhouse, but the infection was much less abundant than was induced on leaves of the same variety with the same inoculum. Infection was secured on larger sizes of fruit in the moist chambers than on the trees. There was a tendency for a small proportion of uninoculated fruits, especially very small ones, to form spongy intumescences at the wounds that resembled early stages of canker, but did not develop into typical cankers. Microscopic examination of these showed the absence of bacterial ooze, and platings from them gave negative results for the presence of the canker organism. The inoculated fruits developed similar intumescences in greater numbers, and these always gave abundant evidence of bacterial invasion, both by microscopic examination and by plating, and the cankers in time developed to typical stages.

In the moist chambers there was the same tendency for the larger fruits to develop very slight external cankers, even though the bacteria had multiplied considerably in the tissues. This was determined by microscopic examination, by plating, and by inoculation of punctured grapefruit leaves with dilutions from the suspected lesions.

In one experiment fruits of several varieties and of uniform size for each variety were used in the moist chambers with three grades of inoculum indicated as I/1, I/20, and I/400. Some of the fruits receiving the I/1 inoculum were held in a refrigerator at about 45° F. The results are shown in Table 10.

TABLE 10.—*Percentages of infection developing on wounded citrus fruits kept in moist chambers after inoculation with Pseudomonas citri, using different dilutions of inoculum*

| Days after inoculation | Fruits kept at room temperature (70° to 75° F.) after inoculation with dilution of inoculum indicated | | | | | | | | | | | | | | | | | | Fruits kept in refrigerator (45° F.) after inoculation with I/1 inoculum | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Marsh grapefruit, 55 mm. diameter | | | Royal grapefruit, 53 mm. diameter | | | Pineapple orange, 45 mm. diameter | | | Washington Navel orange, 46 mm. diameter | | | Rangpur lime, 33 mm. diameter | | | Meyer lemon, 38 mm. diameter | | | Marsh grapefruit | Royal grapefruit | Pineapple orange | Washington navel orange | Rangpur lime | Meyer lemon |
| | I/1 | I/20 | I/400 | I/1 | I/20 | I/400 | I/1 | I/20 | I/400 | I/1 | I/20 | I/400 | I/1 | I/20 | I/400 | I/1 | I/20 | I/400 | | | | | | |
| | *P. ct.* | *P. ct.* | *P. ct.* | *P. ct.* | *P. ct.* | *P. ct.* | *P. ct.* | *P. ct.* | *P. ct.* | *P. ct.* | *P. ct.* | *P. ct.* | *P. ct.* | | *P. ct.* | *P. ct.* | *P. ct.* | *P. ct.* | *P. ct.* | *P. ct.* | *P. ct.* | *P. ct.* | *P. ct.* | *P. ct.* |
| 7 | 70 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 11 | 80 | 30 | 20 | 10 | 2 | 0 | 15 | 0 | 4 | 2 | 0 | 0 | 20 | 10 | 0 | 5 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 15 | 85 | 30 | 20 | 15 | 2 | 0 | 15 | 0 | 10 | 2 | 0 | 0 | 30 | 20 | 0 | 15 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 21 | 90 | 40 | 30 | 15 | 2 | 0 | 15 | 2 | 10 | 2 | 0 | 5 | 30 | 20 | 0 | 15 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 26 | 90 | 40 | 30 | 15 | 2 | 0 | 15 | 2 | 10 | 2 | 0 | 5 | 30 | 20 | 0 | 15 | 0 | 0 | 20 | 0 | 0 | 0 | 10 | 0 |
| 33 | 90 | 40 | 30 | 20 | 4 | 0 | 15 | 2 | 10 | 2 | 0 | 5 | 30 | 20 | 0 | 15 | 0 | 0 | 35 | 0 | 0 | 0 | 15 | 0 |
| 39 | (a) | 40 | (a) | 20 | 5 | 2 | 30 | 2 | (a) | (a) | 0 | ) | 50 | 20 | 2 | 20 | 0 | 0 | 50 | 0 | 0 | 0 | 15 | 0 |
| 52 | ---- | (a) | ---- | 20 | 5 | 2 | (a) | (a) | ---- | ---- | (a) | ---- | (a) | (a) | 2 | (a) | 0 | 0 | 50 | 0 | 0 | 0 | 15 | 0 |
| 61 | ---- | ---- | ---- | (a) | 5 | 2 | ---- | ---- | ---- | ---- | ---- | ---- | ---- | ---- | ---- | ---- | (a) | ---- | (a) | 0 | 0 | 0 | ---- | 0 |
| 80 | ---- | ---- | ---- | ---- | 5 | 2 | ---- | ---- | ---- | ---- | ---- | ---- | ---- | ---- | ---- | ---- | ---- | ---- | ---- | 0 | 0 | 0 | 20 | 10 |
| 98 | ---- | ---- | ---- | ---- | (a) | 2 | ---- | ---- | ---- | ---- | ---- | ---- | ---- | ---- | ---- | ---- | ---- | ---- | ---- | 2 | (a) | ---- | 20 | 10 |
| 120 | ---- | ---- | ---- | ---- | ---- | ---- | ---- | ---- | ---- | ---- | ---- | ---- | ---- | ---- | ---- | ---- | ---- | ---- | ---- | 5 | ---- | ---- | 20 | 10 |

[a] Decayed.

Where strong inoculum was used the infection was evident sooner and developed in a larger percentage of wounds than where weaker inoculum was used. Marsh grapefruit developed lesions more quickly and extensively for each grade of inoculum than did Royal grapefruit of approximately the same size; and Pineapple orange gave similar evidence of having greater susceptibility than Washington Navel orange. Marsh grapefruit was first in apparent susceptibility, and Rangpur lime was second at both room temperature and in the refrigerator. The final reaction developed with I/1 at low temperature was about equal to that developed with I/20 inoculum on the same kind of fruit at room temperature of about 70° to 75° F., but the time required to develop such reaction was much longer at the lower temperature. In other words, at the lower temperature a much stronger inoculum as well as a much longer time was required to produce a given result.

The relative development of canker lesions on wounded fruits of grapefruit and orange is shown in Table 11. This test was made in moist chambers at room temperature, and three dilutions of inoculum were used. Observations were made over a 51-day period. The averages given are for five fruits in each treated lot. In Table 11 the term "blister" is used for any definite infection that had not become erumpent, and so it may have included the pimple or even the watery stage.

TABLE 11.—*Percentages of canker lesions developing on wounded green grapefruits and oranges held in moist chambers after being inoculated with three dilutions of inoculum*

| Days after inoculation | Lesions produced on grapefruits (80 mm. average diameter) with dilution of inoculum indicated | | | | | | Lesions produced on oranges (55 mm. average diameter) with dilution of inoculum indicated | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | I/1 | | I/20 | | I/400 | | I/1 | | I/20 | | I/400 | |
| | Erumpent | Blister | Erumpent | Blister | Erumpent | Blister | Erumpent | Blister | Erumpent | Blister | Erumpent | Blister |
| | *P. ct.* | *P. ct.* | *P. ct.* | *P. ct.* | *P. ct.* | *P. ct.* | *P. ct.* | *P. ct.* | *P. ct.* | *P. ct.* | *P. ct.* | *P. ct.* |
| 14 | 14 | 26 | 1 | 14 | 0 | 0 | 1 | 1 | 0 | 0 | 0 | 0 |
| 20 | 32 | 19 | 4 | 15 | 0 | 2 | 3 | 7 | 0 | 0.6 | 0 | 0.4 |
| 28 | 46 | 19 | 18 | 27 | 6 | 8 | 6 | 21 | 0 | 6 | 0 | 1 |
| 38 | 47 | 25 | 28 | 30 | 8 | 14 | 12 | 20 | 1 | 11 | 0 | 3 |
| 51 | 47 | 27 | 28 | 30 | 9 | 15 | 12 | 26 | 1 | 18 | 0 | 3 |

The fruits were rather large, having been removed from the trees in August. The total amount of reaction in this moist-chamber test is much greater than developed in greenhouse tests of fruits of comparable size. The usual decrease in reaction with dilution of inoculum is noted. Oranges reacted much less in both amount and degree than did grapefruits. To produce a given effect on these oranges required an inoculum more than twenty times as strong (perhaps fifty to one hundred times as strong) as was required for the grapefruits. This is in keeping with the general results shown in Table 10 for smaller fruits.

In other moist-chamber tests Royal grapefruit developed canker less readily than Marsh, Duncan, and seedling grapefruit or shaddock, and Washington Navel oranges developed canker less readily than

Pineapple, Parson Brown, or Valencia. Fruit of Satsuma orange developed slight infection, even under most severe conditions. The results with King and Mandarin oranges were indefinite because of the early rotting of the fruit. Genoa lemon was on a par with Meyer lemon, and Villafranca and Kenedy reacted even less readily. Key lime fruits developed canker readily. Calamondin and kumquat decayed too quickly to give results.

The moist-chamber tests with wounded fruits were started in April and continued until August, successive lots of fruit being sent two or three times a month. The very smallest fruits used decayed too rapidly for canker to develop. Sizes of 12 to 25 mm. could be held long enough to show at least the beginnings of canker lesions. No infections were noted on fruits of such size range for any of the fruits tested. The smallest sizes to show infection in the various tests were as follows: Seedling grapefruit, 50 mm.; Marsh grapefruit, 54 mm.; Royal grapefruit, 53 mm.; Duncan grapefruit, 50 mm.; shaddock, 80 mm.; Pineapple orange, 40 mm.; Parson Brown orange, 41 mm.; Valencia orange, 38 mm.; seedling orange, 28 mm.; Satsuma orange, 35 mm.; key lime, 30 mm.; Buena Vista lime, 30 mm.; Rangpur lime, 33 mm.; Villafranca lemon, 38 mm.; Genoa lemon, 36 mm.; Kenedy lemon, 38 mm.; Meyer lemon, 38 mm.; Sampson tangelo, 35 mm.; King and Mandarin oranges and calamondin decayed too rapidly for infection to occur.

Not all of the above-named varieties were tested in every series, but those most important commercially were tested certainly once and often twice a month. The inoculum was regularly tested on wounded grapefruit leaves and was always potent enough for 100 per cent infection on these. There was certainly abundant opportunity for smaller sizes to become infected. Stomatal infections were very rare.

Mention has already been made of the practical failure to secure a canker reaction in fully matured fruits of orange, lemon, and grapefruit from the market. The large green fruits of orange and grapefruit in August gave strong reactions in moist chambers. On the whole, reaction was stronger for larger sizes of green fruit in the moist chambers than for similar sizes on the trees, and reaction started at larger sizes in the former case than in the latter. No ready explanation of this shifting suggests itself.

In the moist-chamber tests there was abundant multiplication of bacteria in tissue that did not react visibly, as was shown by using graded inoculum on punctured grapefruit leaves. But extensive tests to determine the rate of such increase and its limits as to size of inoculated fruits were not undertaken in the moist-chamber tests. Often a section cut through the inner peel showed a watery infiltration and a slight change in color, suggesting invasion by the bacteria, which microscopic examination confirmed, even when there was no external reaction.

It has been noted that a larger percentage of infections regularly develop in leaf wounds than in fruit wounds, when the same inoculum is used. It might be supposed that the inoculum penetrates less readily to the interior of the deep fruit punctures than to the open leaf punctures. To test this possibility detached wounded fruits were punctured and immersed in the inoculum under an exhaust so as to

withdraw air and cause thorough penetration. These fruits did not develop in moist chambers more infection than those inoculated in the ordinary way, and in both cases the infection was much less than in leaves inoculated at the same time with the same inoculum.

## SUMMARY

A quantitative method is described for estimating from wound inoculations the number of *Pseudomonas citri* present at various stages in the development of canker lesions.

When punctures are made into the oil glands of citrus fruits, infection by *Pseudomonas citri* is seriously hindered. For dependable infection results the oil glands must be avoided in wounding for inoculation tests.

Fruit wounds as much as 8 hours old become infected much less readily than freshly made wounds. If allowed to dry the wounds decrease in infectibility more rapidly than if kept moist.

Weak grades of inoculum produce lower percentages of infection than strong grades, and the resulting cankers begin to show after a longer interval and develop more slowly. With a given strength of inoculum, infection is greater in amount and in degree on wounded leaves than on wounded fruits of a given citrus species.

The size of the fruit influences the amount and degree of canker development. It is difficult to secure wound infections on very small fruits, partly, perhaps, because of the damaging effect of the wounding and subsequent inoculation treatment. Intermediate sizes, from perhaps 25 to 35 mm. in diameter, show best development of canker lesions. Larger fruits develop lesions less readily. The exact upper and lower size limits of susceptibility vary with conditions and can not be inferred from the data at hand.

The majority of infections occur at visible wounds. The so-called stomatal infections occur most abundantly on somewhat smaller sizes of growing fruits than do wound infections, and stomatal infections fail to develop at an upper size range some 10 mm. less than for wound infections.

Periodical testing of infected wounds indicates that the multiplication of the canker bacteria takes place in about equal degree regardless of the size of fruit up to a stage of full maturity on the trees. Fully ripe fruit inoculated through wounds after removal from the tree did not give evidence of any definite increase of the canker organism. A practically maximum number of bacteria is reached in a few days at ordinary temperatures, after which a rather uniform level is maintained for a considerable time. The multiplication of bacteria in the tissues is independent of the development of external evidences of canker. In many instances where there was no visible symptom of canker the bacteria had multiplied just as freely as where there was normal canker development. Such a condition may be referred to as quasi immunity or quasi resistance.

A maximum development of bacteria in lesions may result from weak as well as from strong inoculum, a slightly longer period being required in the former case.

Development of Penicillium rot has a decided inhibiting or killing effect on *Pseudomonas citri* in recently developed lesions in the fruit peel.

Various types and varieties of citrus fruits show differences in the readiness with which either wound or stomatal infections occur. The significance of this in judging relative susceptibility is lessened by the fact that host-tissue reaction may be lacking even when bacterial invasion and development have been at a maximum. The reactive condition of the host tissues is the important factor in canker development.

Tests for persistence of viable canker organisms indicate that they may die out within a period of five or six months, but under certain conditions they may persist for possibly longer periods.

Inoculation tests of limited scope on oranges under orchard conditions indicate general agreement with the findings from experiments in the greenhouse.

Infection tests of green fruits removed from trees and held in moist chambers also confirmed in essentials the findings from the greenhouse experiments. The principal differences were that fruits of the larger sizes developed visible canker lesions rather more readily than did those in the greenhouse tests and that infectibility started at a higher range of size.

At about 45° F. green inoculated fruit in a moist chamber required much stronger inoculum and a much longer time to develop approximately the same visible reaction as similar fruit held at a room temperature of about 70° to 75° F.

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